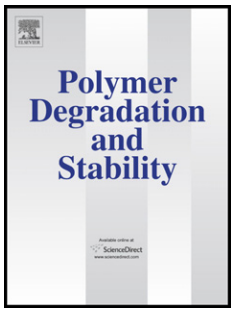

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GLYCEROL PLASTICISED CHITOSAN: A STUDY OF BIODEGRADATION VIA CARBON DIOXIDE EVOLUTION AND NUCLEAR MAGNETIC RESONANCE

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Abstract

The biodegradation of neat chitosan, glycerol plasticised chitosan films and their corresponding clay-based nano-biocomposites has been studied in simulated aerobic soil and composting environments using a respirometric method. The rate of biodegradation was much faster in soil and all test samples achieved close to 100% biodegradation within 70 days. During biodegradation under aerobic composting conditions the neat chitosan samples achieved approx 65% biodegradation and the plasticised chitosan samples achieved >85% biodegradation within 180 days. Additionally, nano-clay additives had no significant effect on the overall biodegradability of the chitosan-based materials during composting. High-resolution solid-state NMR studies were performed to examine the chemical structures of the plasticized chitosan and their nano-biocomposites. NMR studies indicated that the glycerol plasticizer was extracted into wet compost within first few days while acetic acid remained through strong hydrogen bonding with chitosan during the degradation process.

Key words: biodegradation, chitosan, nanoclay, plasticised chitosan, solid-state NMR, nano-biocomposite

1. Introduction

Chitosan, a deacetylated form of chitin, is a natural polymer with repeating units of *β*-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). Chitosan has applications in agriculture, biomedical and drug delivery systems due to its antimicrobial [1-3], biocompatibility [4,5] and good mucoadhesive properties [4]. It also possesses huge potential as a natural, renewable and biodegradable alternative to petroleum-based plastics. Chitosan-based films have gained popularity in e.g., the food packaging industry, especially as edible films or coatings [6,7]. These films are suggested to improve food conservation and quality by forming a barrier against moisture [8], oxygen [9] and CO_2 [10]. These film properties depend on several parameters such as the chitosan molecular weight and the degree of deacetylation, the organic acid used for its solubilisation and the possible presence of plasticizer.

Generally, two methods are used to produce films based on polysaccharides; i) the solvent casting method – despite certain limitations, at the present the only process capable of producing chitosan films; and ii) the melt processing method of extrusion and kneading under thermo-mechanical treatment with plasticizers. The melt processing method is usually preferred for large-scale production of polymeric films, although its adaptation for processing polysaccharides based materials remains challenging. Chitosan, like many other polysaccharides, has very low thermal stability, degrades without melting and thus is considered infusible. We have previously developed and reported a combinatorial and innovative melt-processing route to produce plasticized chitosan sheets using glycerol [11]. The present study aims at investigating biodegradation properties of these materials under simulated aerobic soil and composting conditions.

Addition of nanoclay to natural polymers to form nano-biocomposites, is known to influence the material behavior such as mechanical properties, water absorption, fire resistance and biodegradability [12-18]. In the present study, the effect of addition of nanoclay (un-modified and organically modified) on biodegradability of chitosan-based nano-biocomposites films was investigated under simulated aerobic composting conditions according to standard test method AS ISO 14855-1. The chemical structures of the biodegradation residues were examined by high-resolution solid-state NMR spectroscopy.

2. Materials and Methods

Two types of chitosan were used in the experimental work and their characteristics are shown in Table 1. ChitoClear® was received as a white powder (91% dry matter content) with particle diameter <1 mm (100% through mesh 18). KiOnutrime-Cs® was provided as sandy brown colour powder (92% dry matter content) and with particle diameter <1mm. ChitoClear® was used as the matrix of chitosan-based nano-biocomposites, while KiOnutrime-Cs® was used as the organomodifier for the nanoclay. The Dellite® LVF sodium montmorillonite (MMT–Na^+) was supplied by Laviosa Chimica Mineraria S.p.A. (Italy) and has a cationic exchange capacity (CEC) of 1050 μequiv/g. Glycerol (99.5% purity, from Novance, France), acetic acid (Fluka, Sigma-Aldrich), and sodium hydroxide (Carlo Erba Réactifs – SdS, France), and sodium bromide (Sigma-Aldrich) were used as received.

2.1. Sample preparation

2.1.1. Organomodification of montmorillonite

Chitosan solution was prepared by adding 4.754 g (dry basis) of the KiOnutrime-Cs chitosan to 500 mL of 1% (v/v) acetic acid. The solution was stirred at room temperature overnight. The pH of the solution was then adjusted to 4.9 with NaOH solution. In parallel, a stock of well-dispersed clay suspension was prepared by adding 20 g of MMT–Na^+ into 500 ml of water and treating with sonication at 60 °C for 4 h. Then, the chitosan solution and the MMT–Na^+ suspension were mixed together and the mixture was stirred at 60 °C for 24 h. The mixture was centrifuged at 3000 rpm for 15 min, and then the supernatants were discarded. The precipitate was washed with distilled water and centrifuged again at the same condition, which was repeated twice to make it free from acetate. Hence, the final paste of chitosan-organomodified MMT was obtained with moisture content of 94.6%. Here, the mass ratio of chitosan and clay were thus determined to achieve a monolayer of chitosan absorbed into the nanoclay interlayer spacing through a cationic procedure with respect to the CEC of the nanoclay [19].

2.1.2. Preparation of chitosan-based nano-biocomposites

The preparation procedure of chitosan-based nano-biocomposites used here was similar to that in a previous work, including modifications relating to the addition of nanoclay

[11]. Seven samples with different formulations and/or preparation methods were prepared, with the details listed in Table 2. In summary, glycerol was manually mixed with the chitosan powder and then acetic acid aqueous solution (3%, v/v) was added to the glycerol/chitosan mixture. For unplasticised formulation, acetic acid was directly added to the chitosan powder and mixed. For nano-biocomposites, nanoclay (in the form of either paste or dried powder) was added to glycerol/chitosan mixture first, manually mixed and then acetic acid aqueous solution (3%, v/v) was added to the chitosan–glycerol–nanoclay mixture with continuous mixing to obtain a paste with final chitosan concentration of 25 wt%.

The mixtures with different formulations were then thermo-mechanically kneaded in a Haake Rheocord 9000 internal batch mixer with twin roller rotors at 80 °C for 15 min, with a rotor speed of 100 rpm. The resulting materials were compression moulded at 110 °C under a pressure of 160 bar for 15 min (with a venting process after 8 min), then immediately cooled at room temperature for 5 min. After compression moulding, chitosan sheets of approximately 2 mm thickness were obtained. The sheets were then conditioned in desiccators at 57% relative humidity (achieved with saturated NaBr solution) and ambient temperature for one month to achieve the equilibration of the materials with constant moisture contents. The films were successfully made for all formulations (with or without glycerol) however the unplasticised chitosan films shrank a lot and became curled and rigid during ageing. Detailed properties of all these films will be published in a separate paper (draft under preparation).

2.2. Source of inoculum

The soil sample was collected from a grass field covered with grass, mainly *Pennisetum clandestinum* Kikuyu with some local weeds (CSIRO Highett campus, Victoria, Australia). No herbicide or pesticide has been reportedly used and the site has not been under use or any construction over the past years. Samples were collected and sieved through 8 mm sieve, and a subsample was sent to ALS laboratories (Victoria, Australia) for analysis. The soil characteristics were pH 5.6, dry weight 82%, volatile solids 6.7% and C/N ratio of 13 on an oven-dry basis.

Approximately 2–3 months old mature compost samples were collected from a commercial composting facility (Natural Recovery Systems, Victoria, Australia). It is an in-vessel composting facility which has been converting kerbside garden organics and a range of commercial and industrial food wastes into high quality compost for over a decade. The

compost sourced was sieved using a screen size of 8 mm to obtain a homogeneous mix, free from large inert objects such as glass, stones or pieces of metals. A subsample was sent to ALS laboratories (Victoria, Australia) for analysis. The compost characteristics were pH 7.5, dry weight 52%, volatile solids 44% of dry weight, and C/N ratio of 10 on an oven-dry basis.

2.3. Aerobic biodegradation

The test films were analysed for total dry solids, volatile solids and total organic carbon content and values were used (1) to calculate quantity of material to be used in the test so as to yield suitable amount of carbon dioxide for the determination, and (2) to calculate theoretical amount of carbon dioxide (Equation 1), which is used to determine biodegradation percentage (Equation 2).

Prior to the testing, the film samples were reduced in size to achieve approximately 2 cm × 2 cm maximum surface area of each individual piece of the test material. The test was conducted in triplicate including the blank (soil/compost only), test material (inoculum with test material) and reference material (inoculum with cellulose). Biodegradation experiments under simulated soil environment consisted of 'blank' bioreactor (3L glass vessel) containing approximately 800 g soil on dry weight basis. The 'test' bioreactors contained 800 g of soil and 10 g of test material, both on dry weight basis, and test material was replaced with cellulose in the case of the 'reference' bioreactors. The contents of all bioreactors were well mixed and placed inside an in-house built respirometer unit [20]. The temperature was maintained at 30±2 °C throughout the test.

Biodegradation under aerobic composting environment consisted of 'blank' bioreactors each contained 600 g of total dry solids of compost inoculum. The bioreactors filled with 'test material' each contained 600 g of total dry solids of compost inoculum and 100 g of dry solids of test material and the 'reference' bioreactors were filled with 100 g of cellulose powder and 600 g total dry solids of compost inoculum. These contents were mixed thoroughly before being filled into each replicate bioreactor. All bioreactors were then placed inside an in-house built respirometric unit and the temperature was maintained at 58±2 °C for a maximum period of 180 days.

Aerobic conditions were maintained by providing continuous supply of sufficient airflow to the composting vessels and, to compensate for the water loss, the contents were hydrated and mixed well once a week. The amount of CO_2 generated in each bioreactor was measured (at least twice a day) using an infra-red CO_2 analyser and values were data logged into the

computer. Visual observations were recorded, pH was measured and digital photos were taken at regular intervals.

Theoretical amount of carbon dioxide $THCO_2$, in grams per bioreactor, that the test material can produce, was calculated using following Equation 1:

$$THCO_2 = M_{TOT} \; x \; C_{TOT} \; x \; \frac{44}{12}$$

where, M_{TOT} is the total dry solids (in grams) in the test material at the start of the test; C_{TOT} is the proportion of total organic carbon in the total dry solids in the test material (in grams per gram); 44 and 12 are the molecular mass of carbon dioxide and the atomic mass of carbon, respectively.

Equation 1: Equation for determining theoretical carbon dioxide

To monitor the biodegradation process, cumulative amounts of carbon-dioxide released from each bioreactor each month were compiled in a tabular form and percentage biodegradation D_t was calculated (for the test material and the reference material) for each point of time when the measurements were made using Equation 2:

$$D_t = \frac{(CO_2)_T - (CO_2)_B}{THCO_2} x100$$

where, $(CO_2)_T$ is the cumulative amount of carbon dioxide evolved in each bioreactor containing test material (in grams per bioreactor); $(CO_2)_B$ is the mean cumulative amount of carbon dioxide evolved in the blank vessel (in grams per bioreactor)

Equation 2: Equation for determining percentage biodegradation of materials

Following this step, the cumulative amount of carbon dioxide evolved as a function of time and a curve of percentage biodegradation as a function of time were plotted. Mean values were calculated from the replicate bioreactors (which showed difference of <20% between individual values) and used for plotting the curves. The test was terminated after duration of 160 days.

2.4. Scanning electron microscopy (SEM) and solid-state Nuclear Magnetic Resonance (NMR)

Film samples were collected at regular intervals during biodegradation period. Specimens were gently rinsed with sterilised distilled water, air-dried overnight and their surfaces were coated (to approximately 300Å) with iridium prior to examination, then

examined under high vacuum by a scanning electron microscopy (SEM) using Philips FEI XL-30 SFEG. The electron beam with an accelerating voltage of 5 kV was used to produce high definition images.

High-resolution solid-state NMR experiments were conducted at room temperature using a Varian 300 NMR Systems at resonance frequencies of 75 MHz for ^{13}C and 300 MHz for ^{1}H. ^{13}C NMR spectra were observed under either CP/MAS/DD (cross polarization, magic angle spinning, and high power decoupling) conditions or using a single 90° pulse excitation (SPE) method with high power decoupling. The 90° pulse was 5.5 μs for ^{1}H and ^{13}C while the spinning rate of MAS was set at a value in the range of 8 – 9 kHz. A contact time of 1.0 ms was used for measuring CP/MAS spectra while the repetition time was 2 s for all measurements. The chemical shift of ^{13}C CP/MAS spectra was determined by taking the carbonyl carbon of solid glycine (176.3 ppm) as an external reference standard.

3. Results and Discussion

Chitosan is known to be susceptible to biodegradation by chitosanase enzyme producing environmental microorganisms present in soil [21,22], marine [23,24] and fresh water systems [25]. However, the biodegradation mechanism involved is not clearly understood. In this study biodegradability of neat chitosan, glycerol plasticised chitosan films and their corresponding clay-based nano-biocomposites was studied in simulated aerobic soil and composting environments using a respirometric method and results are discussed in following sections.

3.1. Aerobic biodegradation in soil

Visual observations were recorded and photographs of chitosan test films were taken during biodegradation in soil (Figure 1). The chitosan samples were covered with white mycelium growth (fungi or actinobacteria) after two weeks of incubation in soil. As the time progressed, chitosan film samples were rapidly biodegraded by soil microorganisms and by end of four weeks, the film fragments could no longer be distinguished from soil. These results are supported by several other research studies that have reported biodegradation of chitosan by soil microorganisms [10,26]. Globally distributed members of genera *Arthrobacter, Aspergillus, Bacillus, Streptomyces, Pseudomonas* and *Penicillium* are known to produce enzymes, chitosanases, which can degrade chitosan [21,22,27,28].

The respirometric biodegradation testing of chitosan in soil has rarely been reported. Chitosan is known to possess antimicrobial properties [29] which could interfere with its biodegradability in natural environments. To investigate the influence of glycerol plasticization on biodegradability of chitosan in soil, the cumulative CO_2 and percentage biodegradation profiles for neat chitosan and plasticised chitosan films samples are shown in Figure 2. A steady rate of cumulative carbon dioxide evolution from composting vessels containing cellulose and chitosan samples (Figure 2A) suggested that these test materials were easily metabolised by soil microorganisms present in the inoculum. A short lag phase was observed for neat chitosan samples during first week, thereafter its rate of biodegradation progressively increased. In comparison, biodegradation of the glycerol plasticised chitosan samples started immediately after incubation in soil and they continued to biodegrade rapidly (Figure 2B). It is likely that relatively short lag phase observed for neat chitosan samples in comparison to plasticised chitosan samples could be the result of antimicrobial activity of chitosan from certain microorganisms present in the soil [1,3,29]. However, as the microbial population acclimatise to their surrounding environment the rate of biodegradation steadily increased. The rapid biodegradability of glycerol plasticised chitosan films suggested that glycerol, a highly biodegradable material [30-33], played an important role in enhancing the rate of biodegradation. It has been reported by several authors that plasticisation with glycerol increases water vapour permeability, thus making films more hydrophilic [34-36] and easily accessible for microbial attack. Besides, the “free” (which are not fully mobilized) glycerol molecules tend to uptake the water molecules by an exchange mechanism with the environment by sorption/desorption, toward the equilibration. The glycerol increases the global water content of the material (Table 1) and then the swelling of the material, which give rise to an increase of the transport phenomena inside the material. The samples with chitosan/glycerol ratio of 75/25 (w/w) biodegraded most rapidly, followed by samples with chitosan/glycerol ratio of 90/10 (w/w) and the neat chitosan. All test samples reached close to 100% biodegradation within 70 days of test period (Figure 2B).

^{13}C SPE/MAS NMR spectra for unplasticised chitosan and glycerol-plasticised chitosan (with 10 or 25% of glycerol) samples, during the first two weeks of biodegradation are shown in Figure 3. Since a short repetition time (2s) was used, mainly the mobile components in the materials were detected, such as glycerol plasticizer, acetic acid, fat or lipid, and plasticised chitosan. After biodegradation for one week, glycerol (sharp peaks at 73 and 64 ppm), acetic acid and lipid (sharp peaks at 175-180 ppm, 20-30 ppm) disappeared from the residual solid. In explanation, we suppose that these mobile components were

mainly extracted (washed out) into the soil rather than fully biodegraded because the biodegradation percentage is still very limited after one week. The remaining resonances are attributed to plasticized chitosan and the intensity decreased significantly by taking background signal (bs) as an internal reference. The line width of these resonances also became broad, indicating that a wide distribution of the chemical structures was formed. On the other hand, the ^{13}C CP/MAS spectra (Figure 4) are sensitive to relatively rigid components, reflecting the chemical nature of the components in the rigid phase. Except the loss of glycerol (peaks 73 and 64 ppm) in the 75chitosan/25glycerol samples within the 1st week biodegradation, all three samples behaved in a similar manner with no significant changes in chemical structures. It is interesting to note that the acetic acid signals (175 and 20 ppm) were also observed in the CP/MAS NMR spectra all the time, suggesting its strong hydrogen bonding with the chitosan segments during the biodegradation.

3.2. Aerobic biodegradation in compost

Visual observations were recorded and photographs are shown in Figure 5 for chitosan samples during biodegradation in compost. After one week of incubation, the chitosan samples swell and they were completely covered with white mycelium growth of compost microorganisms. Over next two weeks all test samples, except neat chitosan, disintegrated rapidly into smaller fragments which were difficult to be distinguished from compost. In comparison, neat chitosan samples retained their structural integrity for a much longer period of time, and slowly started disintegrating only after five weeks of composting.

SEM analysis of samples collected at different time periods are presented in Figure 6. The day ‘0’ samples had an uneven and wrinkly surface that could have resulted from shrinkage of samples during ageing process. As the biodegradation progressed, the surface morphology changed. It was challenging to get rid of the adhering compost and obtain clear images. Samples were briefly rinsed with ethanol prior to sample preparation that could have washed out adhering microbial (bacteria or fungi) cells as no clear proof of microbial growth was observed on samples. Globular structures identified on most sample surfaces during degradation were those of plant spores (*Lycopodium* sp).

The cumulative CO_2 and percentage biodegradation profiles for each test sample are shown in Figure 7. The amount of carbon dioxide evolved depends upon the carbon content and quantity of the test material used in the experiment. Cellulose had higher carbon content as compared to chitosan and a relatively lesser quantity of chitosan samples was used hence the observed difference in their cumulative CO_2 values. Steady rates of carbon dioxide

evolution from each composting vessel indicate that test materials were actively metabolised by microbial population present in the compost (Figure 7A). Similar results were observed by Xu et al [37] during their biodegradation studies on acetylated chitosan films. It was observed that the rate of biodegradation of neat chitosan was relatively much slower than all other samples analysed in this study (Figure 7B). Biodegradation of the chitosan samples with glycerol was initiated immediately after incubation in compost, without any lag phase. As a result, all samples achieved more than 50% biodegradation within the first two weeks of composting. In comparison, neat chitosan reached approximately 18% biodegradation at the end of second week. During week 3, a significant decrease in the rate of biodegradation was observed for all the chitosan samples (but not in the positive reference, cellulose). Previous studies have reported that alkyl amides and their corresponding *N*-derivatives alkyl amines have antimicrobial properties. [38] Based on the results obtained in this study, we hypothesise that microbial activity in the composting vessels containing chitosan samples was significantly influenced in the presence of certain inhibitory substances produced as a by-product during chitosan biodegradation [1,2,39]. As time progressed (i.e. during week 4), inhibitory substances were presumably further degraded into products which were less effective in inhibiting microbial activity or easily susceptible to microbial degradation. As a result, the rate of biodegradation increasingly improved. A steady rate of biodegradation was observed for all test samples until week 8. The neat chitosan samples biodegraded by up to 45% whereas the glycerol plasticised chitosan samples biodegraded 60%–80% at the end of 60 days of composting. During week 9, a slight decrease in the level of biodegradation was observed for all the chitosan samples but not as significant as observed during week 3. A steady rate of biodegradation was observed thereafter and neat and modified chitosan samples achieved respectively approximately 65% and more than 85% biodegradation after 180 days. In the literature [40,41], it has been widely reported that addition of nanoclay to nano-biocomposites results in improved thermal stability, tensile and gas barrier properties but there have been conflicting reports on the effect of clay on their biodegradability. Some studies have reported negative [42] or no significant effect on overall biodegradability [12,43] whereas other studies have reported an improvement in biodegradability of nanocomposites with nanoclays [44,45]. To provide clarity, there is a need to further investigate the role of nanoclay during biodegradation process.

Chiou et al [12] has reported that addition of nanoclay improved resistance to moisture of nano-biocomposites based on starch. Exfoliated/intercalated and dispersed clay creates specific nano and micro structures, which modify the material permeation and the water

absorption thus influencing the rate of biodegradation. Table 1 shows for instance that the addition of (unmodified or organo-modfied) MMT decreases the global water uptake after equilibration. However, in the present study the overall degree of biodegradation of the chitosan samples did not seem to be dramatically affected by addition of the nanoclay (Figure 7). Samples containing the modified nanoclay demonstrated no such effect on biodegradation and this could be probably due to the nano-structuration of the different samples. The unmodified nanoclay samples showed a slight increase in their relative degree of biodegradation due to the inherent defects in the samples from nanoclay agglomeration. These results are in agreement with another recent study by Hsu et al [46] which has reported significant increase in *in-vivo* biodegradability of chitosan–MMT nanocomposites relative to pure chitosan samples.

^{13}C SPE/MAS NMR spectra for chitosan/glycerol 75/25 and its nanoclay composites after biodegradation in compost during first five weeks are shown in Figure 8. Similar to soil degradation behaviour (Figure 3), mobile components glycerol, acetic acid and some mobile plastisized chitosan were observed for the samples before biodegradation. But glycerol signals disappeared after one week biodegradation as it migrated into wet compost. However, the relative intensity of the mobile chitosan signals was much stronger here due to slow aerobic biodegradation in compost. The acetic acid signals were also observed in the spectra up to 80 days biodegradation, indicating its strong hydrogen bonding with chitosan and slow biodegradation rate. ^{13}C CP/MAS spectra were also recorded (data not shown) but did not show significant structure changes up to 80 days biodegradation for both 75chitosan/25glycerol sample and its nanoclay composites.

4. Conclusions

The biodegradation of neat chitosan, chitosan/glycerol 90/10 (wt/wt), chitosan/glycerol 75/25 (wt/wt) and its nanoclay composites have been studied in soil and compost using CO_2 evolution by the respirometric method. The materials reached 100% biodegradation within 70 days in soil, but the biodegradation was much slower under compost conditions. Neat chitosan achieved approx 65% biodegradation and glycerol-modified chitosan samples achieved more than 85% biodegradation respectively after 180 days. NMR analysis revealed that the glycerol plasticizer was extracted into wet compost within first few days while acetic acid remained through strong hydrogen bonding with chitosan along the degradation process.

In the current study, addition of nanoclay to plasticised chitosan samples had no significant effect on their overall biodegradability.

The study presents extensive report on biodegradation of unplasticised chitosan and glycerol-plasticised chitosan in soil and compost environments. The results support application of chitosan-based nano-biocomposites as sustainable materials of the future. Further research is needed into understanding exact role of nanoclay during biodegradation, and identification of microorganisms and enzymes responsible for rapid biodegradation of chitosan-based nano-biocomposites.

References

[1] Badawy MEI, Rabea EI. Characterization and antimicrobial activity of water-soluble N-(4-carboxybutyroyl) chitosans against some plant pathogenic bacteria and fungi. Carbohydrate Polymers 2012;87:250-6.

[2] Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules 2003;4:1457-65.

[3] Guo ZY, Chen R, Xing RE, Liu S, Yu HH, Wang PB, et al. Novel derivatives of chitosan and their antifungal activities in vitro. Carbohydrate Research 2006;341:351-4.

[4] Kumar M, Muzzarelli RAA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chemical Reviews 2004;104:6017-84.

[5] Kumar PTS, Lakshmanan VK, Anilkumar TV, Ramya C, Reshmi P, Unnikrishnan AG, et al. Flexible and Microporous Chitosan Hydrogel/Nano ZnO Composite Bandages for Wound Dressing: In Vitro and In Vivo Evaluation. ACS Applied Materials & Interfaces 2012;4:2618-29.

[6] Cagri A, Ustunol Z, Ryser ET. Antimicrobial edible films and coatings. Journal of Food Protection 2004;67:833-48.

[7] Devlieghere F, Vermeulen A, Debevere J. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiology 2004;21:703-14.

[8] Caner C, Vergano, P. J., & Wiles, J. L. Chitosan film mechanical and permeation properties as affected by acid, plasticizer, and storage. Journal of Food Science 1998;63:1049.

[9] Laufer G, Kirkland C, Cain AA, Grunlan JC. Clay-Chitosan Nanobrick Walls: Completely Renewable Gas Barrier and Flame-Retardant Nanocoatings. ACS Applied Materials & Interfaces 2012;4:1643-9.

[10] Hosokawa J, Nishiyama, M., Yoshihara, K., & Kubo, T. Biodegradable film derived from chitosan and homogenized cellulose. Industrial and Engineering Chemistry Research 1990;29:800.

[11] Epure V, Griffon M, Pollet E, Averous L. Structure and properties of glycerol-plasticized chitosan obtained by mechanical kneading. Carbohydrate Polymers 2011;83:947-52.

[12] Chiou BS, Wood D, Yee E, Imam SH, Glenn GM, Orts WJ. Extruded Starch-Nanoclay nanocomposites: Effects of glycerol and nanoclay concentration. Polymer Engineering and Science 2007;47:1898-904.

[13] Huang MF, Yu JG, Ma XF. Studies on the properties of Montmorillonite-reinforced thermoplastic starch composites. Polymer 2004;45:7017-23.

[14] Park HM, Liang XM, Mohanty AK, Misra M, Drzal LT. Effect of compatibilizer on nanostructure of the biodegradable cellulose acetate/organoclay nanocomposites. Macromolecules 2004;37:9076-82.

[15] Wang SF, Shen L, Tong YJ, Chen L, Phang IY, Lim PQ, et al. Biopolymer chitosan/montmorillonite nanocomposites: Preparation and characterization. Polymer Degradation and Stability 2005;90:123-31.

[16] Gaurav A, Ashamol A, Deepthi MV, Sailaja RRN. Biodegradable nanocomposites of cellulose acetate phthalate and chitosan reinforced with functionalized nanoclay: Mechanical, thermal, and biodegradability studies. Journal of Applied Polymer Science 2012;125:E16-E26.

[17] Singh NK, Das Purkayastha B, Roy JK, Banik RM, Yashpal M, Singh G, et al. Nanoparticle-Induced Controlled Biodegradation and Its Mechanism in Poly(epsilon-caprolactone). ACS Applied Materials & Interfaces 2010;2:69-81.

[18] Avérous L, Pollet E. Environmental Silicate Nano-biocomposites. London: Springer-Verlag May 2012. p. 447.

[19] Darder M, Colilla M, Ruiz-Hitzky E. Biopolymer-clay nanocomposites based on chitosan intercalated in montmorillonite. Chemistry of Materials 2003;15:3774-80.

[20] Way C, Wu DY, Dean K, Palombo E. Design considerations for high-temperature respirometric biodegradation of polymers in compost. Polymer Testing 2010;29:147-57.

[21] Sinha S, Tripathi P, Chand S. A New Bifunctional Chitosanase Enzyme from Streptomyces sp and its Application in Production of Antioxidant Chitooligosaccharides. Applied Biochemistry and Biotechnology 2012;167:1029-39.

[22] Kang LX, Chen XM, Fu L, Ma LX. Recombinant expression of chitosanase from Bacillus subtilis HD145 in Pichia pastoris. Carbohydrate Research 2012;352:37-43.

[23] Shaikh SA, Deshpande MV. Chitinolytic Enzymes - Their contribution to basic and applied research. World Journal Microbiology and Biotechnology 1993;9:468-75.

[24] Kobayashi T, Koide O, Deguchi S, Horikoshi K. Characterization of Chitosanase of a Deep Biosphere Bacillus Strain. Bioscience Biotechnology Biochemistry 2011;75:669-73.

[25] Gooday GW, Prosser JI, Hillman K, Cross MG. Mineralization of chitin in an estuarine sediment - The importance of the chitosan pathway. Biochemical Systematics and Ecology 1991;19:395-400.

[26] Makarioslaham I, Lee TC. Biodegradability of chitin-containing and chitosan-containing films in soil environment. Journal of Environmental Polymer Degradation 1995;3:31-6.

[27] Yoshihara K, Hosokawa J, Kubo T, Nishiyama M. Isolation and identification of a chitosan degrading bacterium belonging to genus Pseudomonas and chitosanase production by the isolate. Agricultural and Biological Chemistry 1990;54:3341-3.

[28] Wei XL, Chen W, Xiao M, Xiao JB, Wang YF. Isolation, Characterization, and Application of Chitosan-Degrading Fungus from Soil. Journal of Microbiology and Biotechnology 2010;20:1114-20.

[29] Goy RC, de Britto D, Assis OBG. A Review of the Antimicrobial Activity of Chitosan. Polimeros-Ciencia E Tecnologia 2009;19:241-7.

[30] Santibanez C, Varnero MT, Bustamante M. Residual glycerol from biodiesel manufacturing, waste or potential source of bioenergy: A review. Chilean Journal of Agricultural Research 2011;71:469-75.

[31] Domenek S, Feuilloley P, Gratraud J, Morel MH, Guilbert S. Biodegradability of wheat gluten based bioplastics. Chemosphere 2004;54:551-9.

[32] Dinkel VG, Frechen FB, Dinkel AV, Smirnov YY, Kalyuzhnyi SV. Kinetics of Anaerobic Biodegradation of Glycerol by Sulfate-Reducing Bacteria. Applied Biochemistry and Microbiology 2010;46:712-8.

[33] Viana MB, Freitas AV, Leitao RC, Santaella ST. Biodegradability and methane production potential of glycerol generated by biodiesel industry. Water Science and Technology 2012;66:2217-22.

[34] Suyatma NE, Tighzert L, Copinet A. Effects of hydrophilic plasticizers on mechanical, thermal, and surface properties of chitosan films. Journal of Agricultural and Food Chemistry 2005;53:3950-7.

[35] Garcia MA, Pinotti A, Zaritzky NE. Physicochemical, water vapor barrier and mechanical properties of corn starch and chitosan composite films. Starch-Starke 2006;58:453-63.

[36] Lamim R, de Freitas RA, Rudek EI, Wilhelm HM, Cavalcanti OA, Bresolin TM. Films of chitosan and N-carboxymethylchitosan. Part II: Effect of plasticizers on their physiochernical properties. Polymer International 2006;55:970-7.

[37] Xu J, McCarthy SP, Gross RA, Kaplan DL. Chitosan film acylation and effects on biodegradability. Macromolecules 1996;29:3436-40.

[38] Kabara JJ, Truant JP, Conley AJ. Relationship of chemical structure and antimicrobial activity of alkyl amides and amines. Antimicrobial Agents and Chemotherapy 1972;2:492-&.

[39] Tikhonov VE, Stepnova EA, Babak VG, Yamskov IA, Palma-Guerrero J, Jansson HB, et al. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives. Carbohydrate Polymers 2006;64:66-72.

[40] Ray SS, Okamoto K, Okamoto M. Structure and properties of nanocomposites based on poly(butylene succinate) and organically modified montmorillonite. Journal of Applied Polymer Science 2006;102:777-85.

[41] Okamoto M. Biodegradable polymer/layered silicate nanocomposites: A review. Journal of Industrial and Engineering Chemistry 2004;10:1156-81.

[42] Lee SR, Park HM, Lim H, Kang TY, Li XC, Cho WJ, et al. Microstructure, tensile properties, and biodegradability of aliphatic polyester/clay nanocomposites. Polymer 2002;43:2495-500.

[43] Sangwan P, Way C, Wu DY. New Insight into Biodegradation of Polylactide (PLA)/Clay Nanocomposites Using Molecular Ecological Techniques. Macromolecular Bioscience 2009;9:677-86.

[44] Okamoto K, Ray SS, Okamoto M. New poly(butylene succinate)/layered silicate nanocomposites. II. Effect of organically modified layered silicates on structure, properties, melt rheology, and biodegradability. Journal of Polymer Science Part B-Polymer Physics 2003;41:3160-72.

[45] Ray SS, Yamada K, Okamoto M, Ogami A, Ueda K. New polylactide/layered silicate nanocomposites. 3. High-performance biodegradable materials. Chemistry of Materials 2003;15:1456-65.

[46] Hsu SH, Wang MC, Lin JJ. Biocompatibility and antimicrobial evaluation of montmorillonite/chitosan nanocomposites. Applied Clay Science 2012;56:53-62.

List of Tables

Table 1. Chitosan source and properties (as provided by the supplier)

Commercial name	**KiOnutrime-Cs®**	**ChitoClear™**
Supplier	KitoZyme	Primex
Source	Aspergillus niger (mushroom)	Pandalus borealis (shrimp)
Molecular Mass	15,000 Da	250,000–300,000 Da
Degree of deacetylation	78–80%	96%

Table 2. Formulations of the different plasticised chitosan-based materials/nano-biocomposites, and water uptake after equilibration.

Samples	Chitosan [a]	Glycerol	3% acetic acid [b]	MMT	OMMT	Moisture content (%) [c]
100 Chitosan	100	0	300	0	0	24.10±0.06
90 Chitosan/ 10 glycerol	90	10	270	0	0	32.40±0.38
75 Chitosan/25 glycerol	75	25	225	0	0	42.20±0.05
75 Chitosan/25 glycerol/5.0 MMT	75	25	225	5	0	27.20±0.11
75 Chitosan/25 glycerol/5.0 modified MMT	75	25	225	0	5	26.20±0.01

All values are in weight portions (unless where specifically stated); [a] on dry base;[b] the actual additions were adjusted considering the moisture content in raw chitosan and that in OMMT; [c] determined by weight loss in oven at 60 °C for 12 days after conditioning at 57% relative humidity for 1 month;

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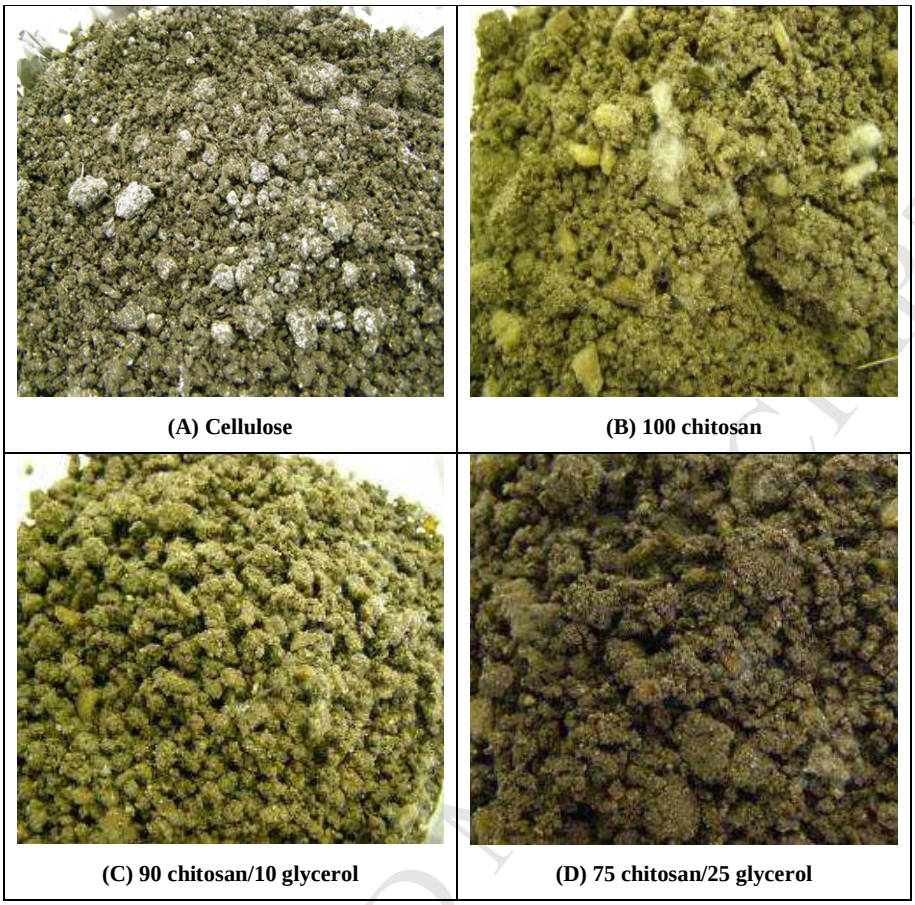
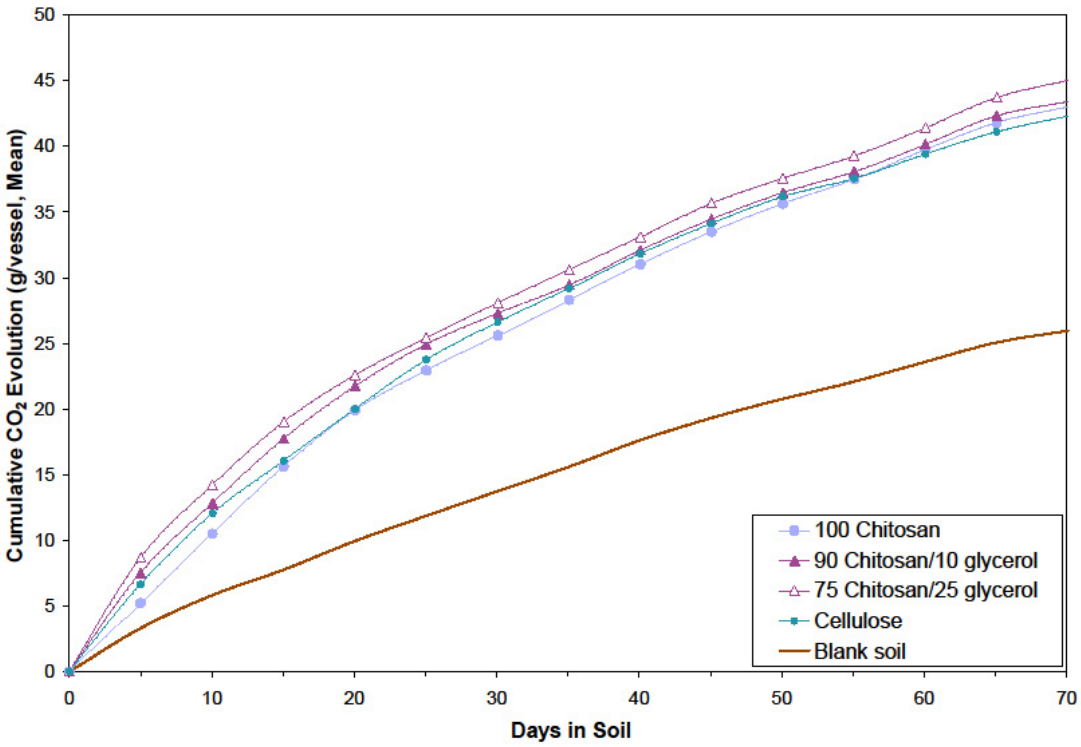


Figure 1. Cellulose and Chitosan samples after two weeks in soil
(NB: The width of photograph for 'A' and 'C' is 10cm, and 'B' and 'D' is 5cm)



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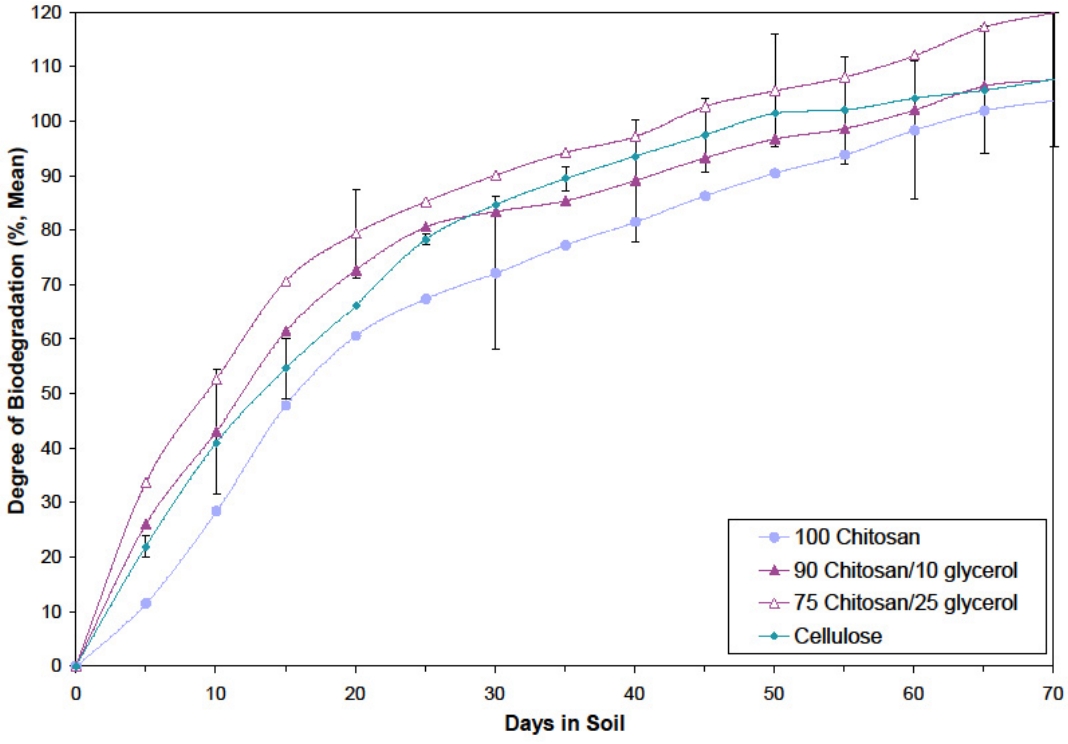


(B)

Figure 2. (A) Cumulative CO2 evolved during aerobic biodegradation in soil and (B) percentage biodegradation in soil.

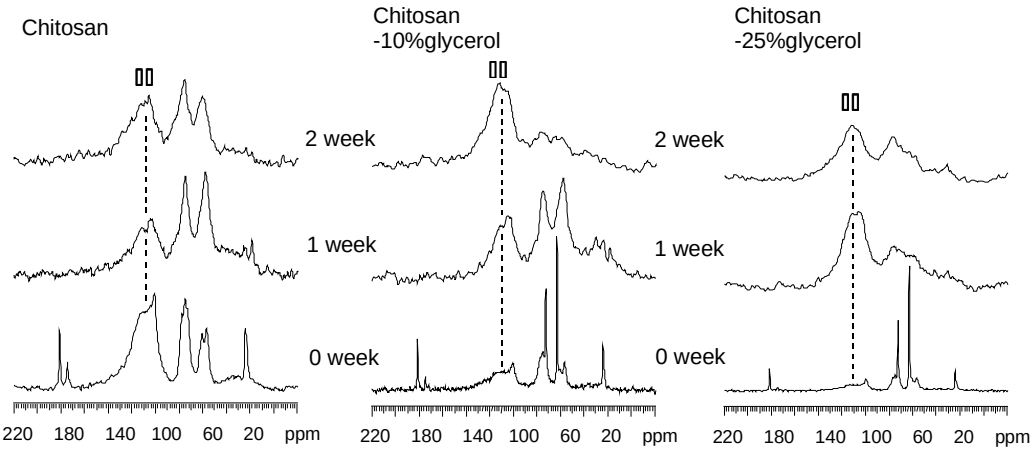


Figure 3. ^{13}C SPE/MAS NMR spectra of neat chitosan, chitosan/glycerol 90/10 (wt/wt) chitosan/glycerol 75/25 (wt/wt) before and after soil biodegradation for 2 weeks. (bs: background signal of the sample spinner).

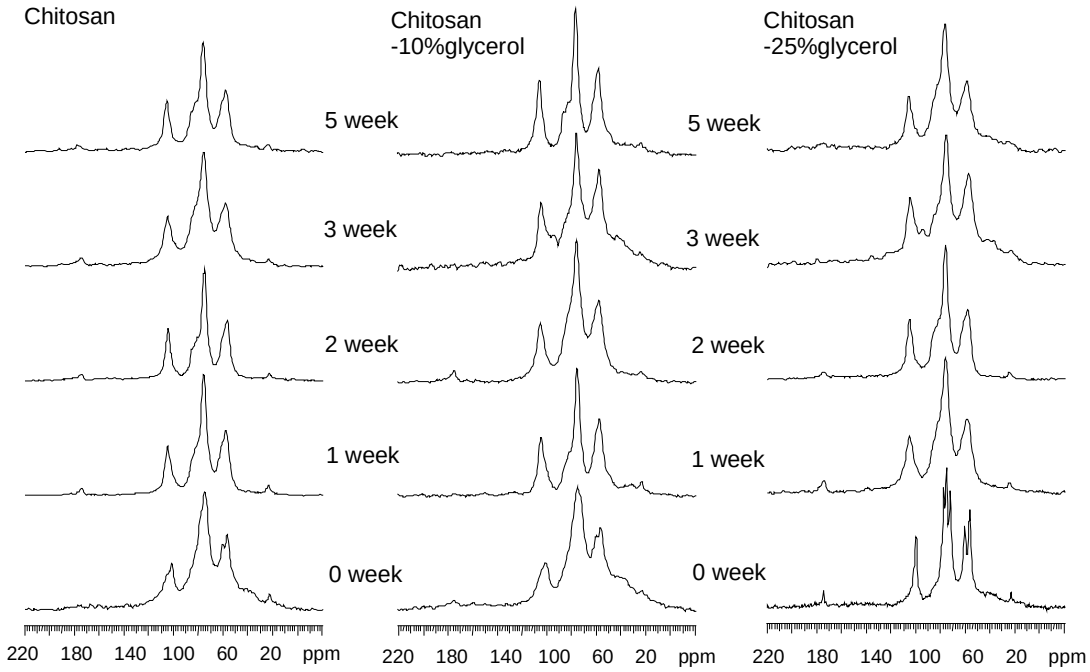


Figure 4. ^{13}C CP/MAS NMR spectra of neat chitosan, chitosan/glycerol 90/10 (wt/wt) chitosan/glycerol 75/25 (wt/wt) before and after soil biodegradation for 5 weeks.

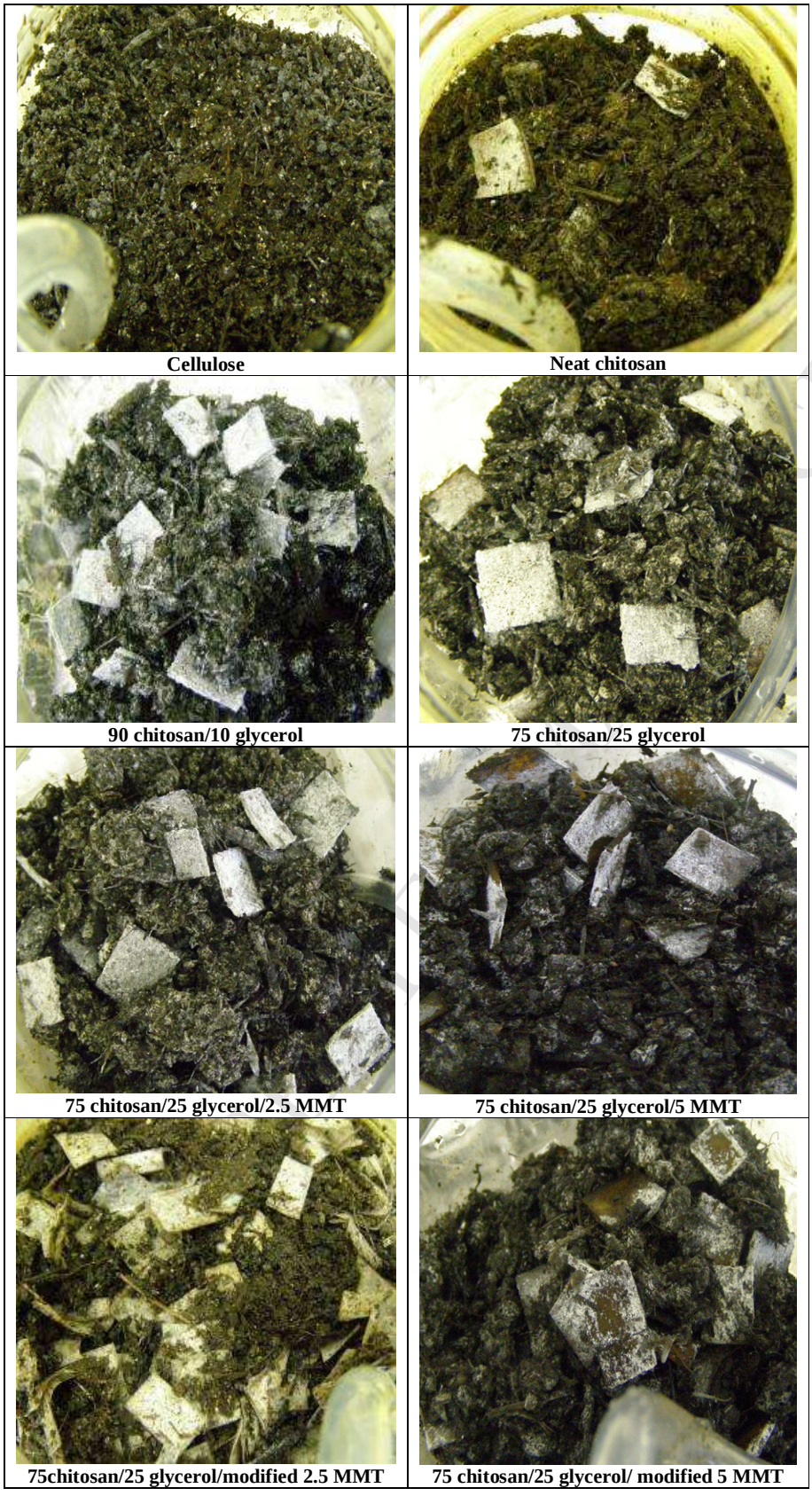


Figure 5. Cellulose reference and chitosan test samples after two weeks of composting. (NB: Approx width of all photographs is 10cm)

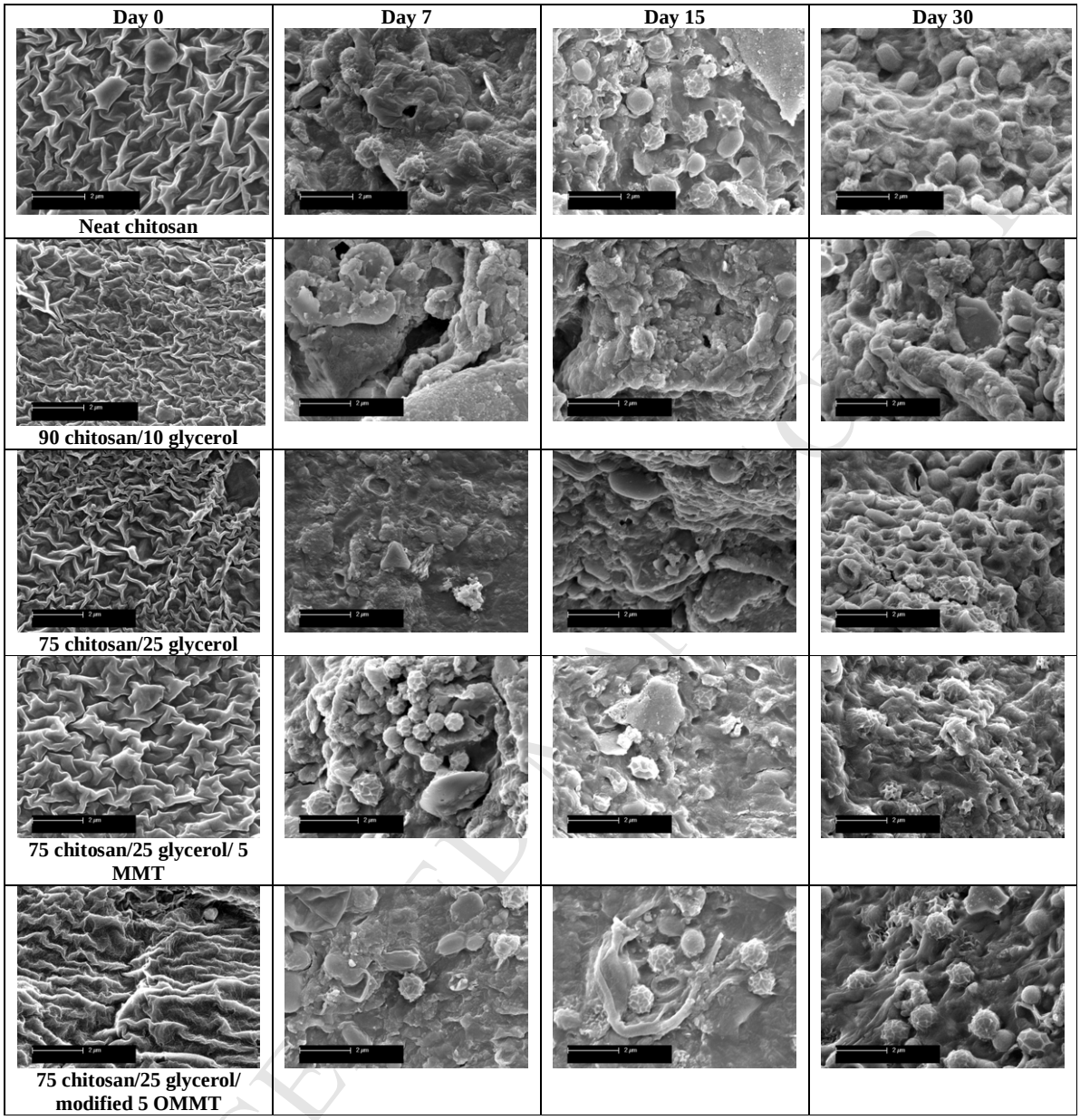
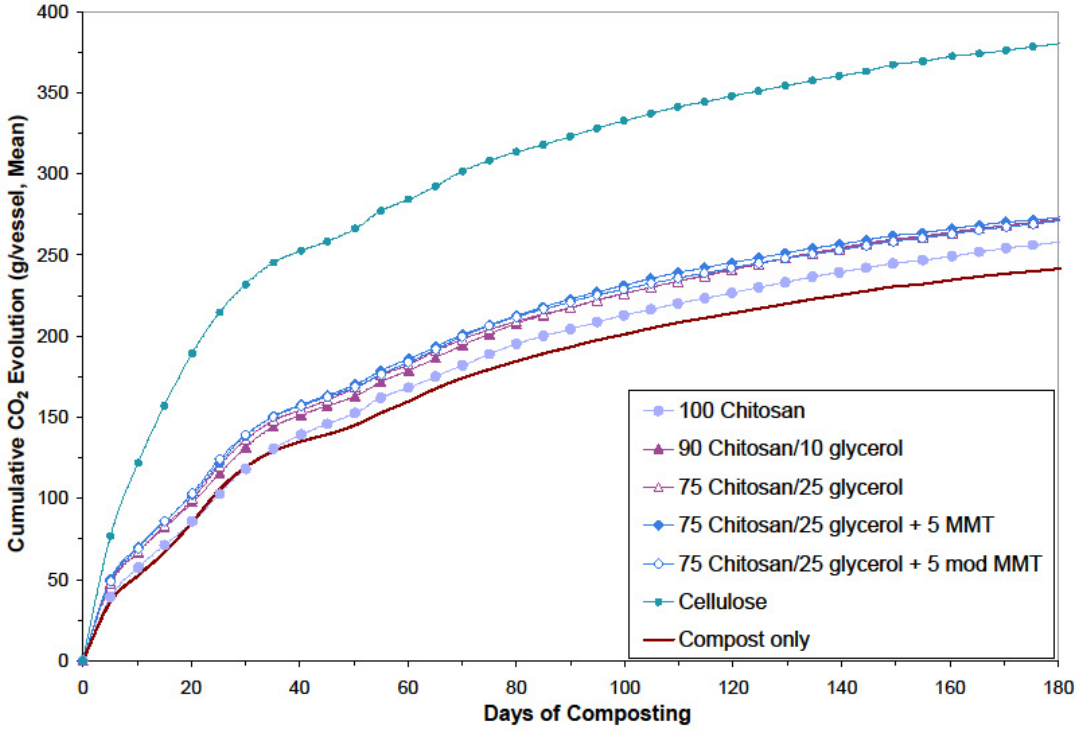


Figure 6. SEM of chitosan samples on day 0 and after 7, 15 and 30 days of biodegradation in compost. Globular structures observed on degraded sample surface are plant spores commonly present in soil/compost (NB: Scale bar for all micrographs is 2 micron)



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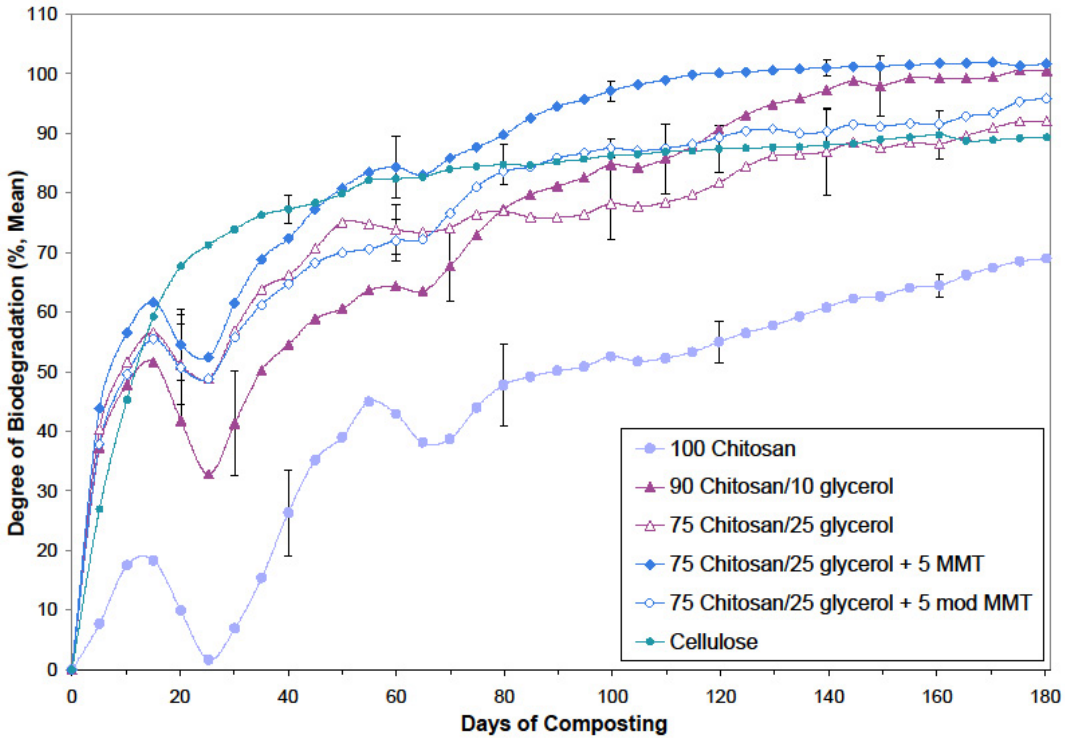


(B)

Figure 7. (A) Cumulative CO_2 evolution during 180 days of aerobic composting and (B) percentage biodegradation during 180 days of aerobic composting.

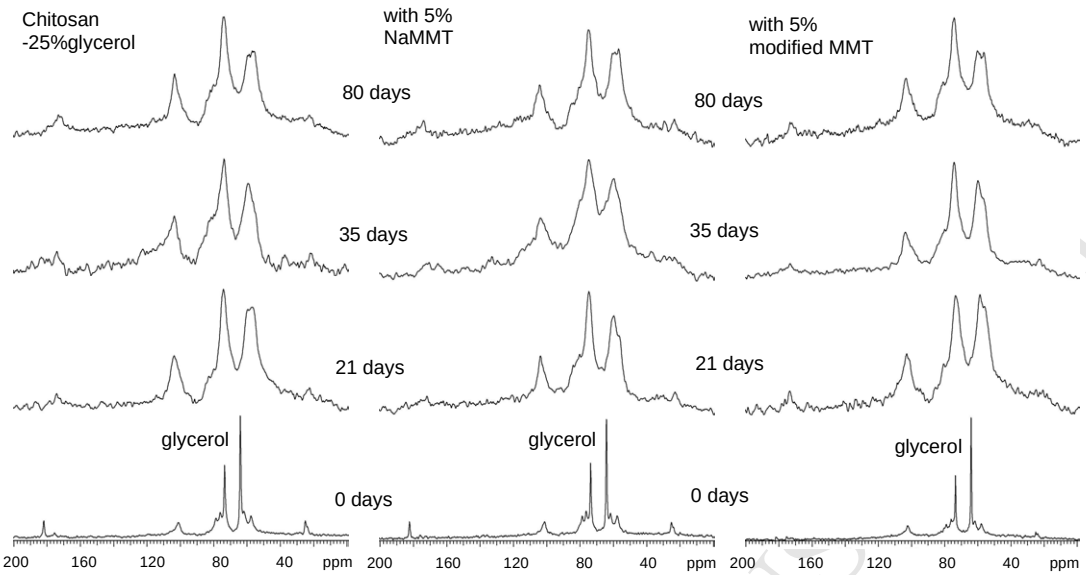


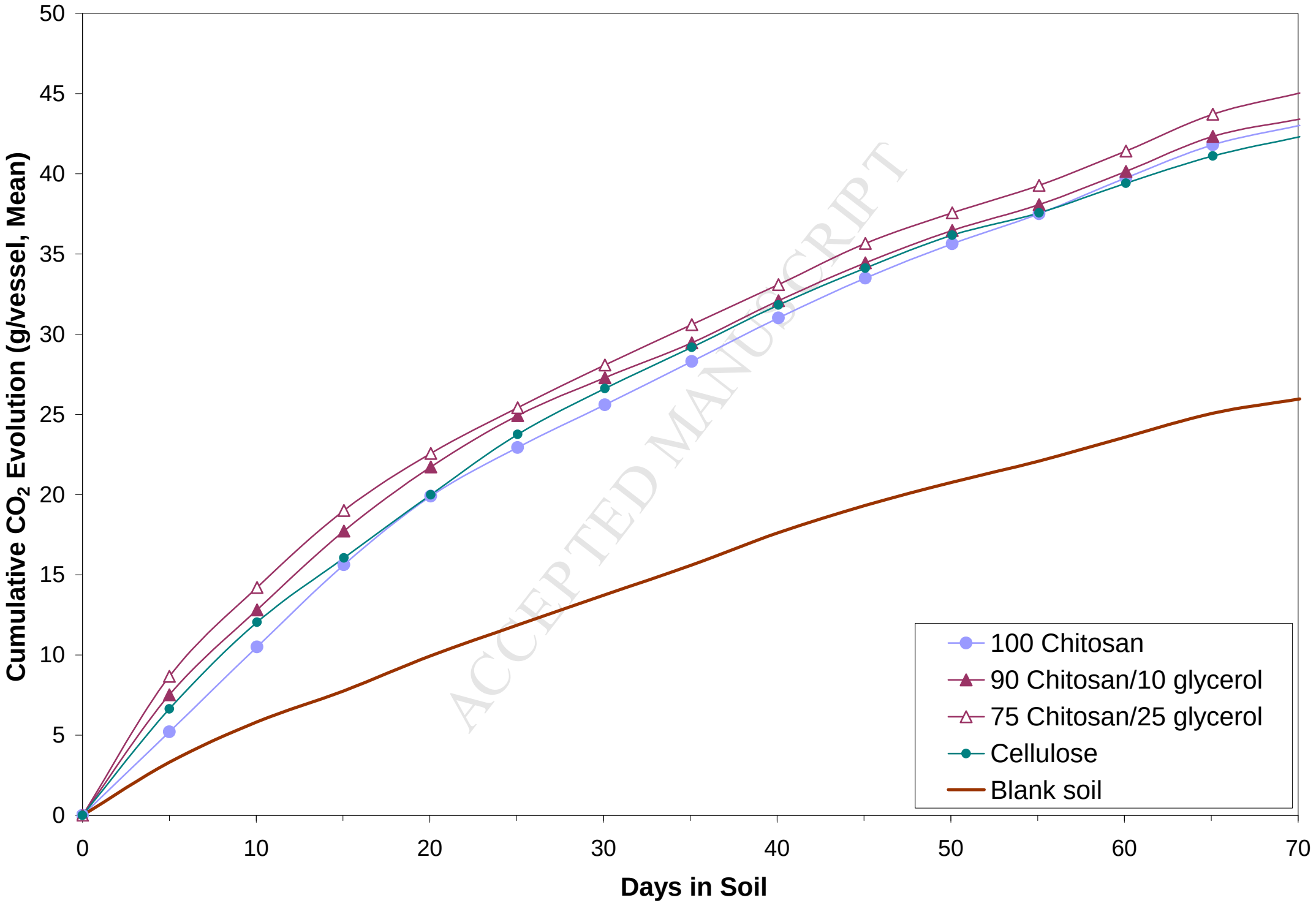
Figure 8. ^{13}C SPE/MAS NMR spectra of chitosan/glycerol 75/25 (wt/wt) and its nanoclay composites before and after aerobic composting for 80 days. (bs: background signal of the sample spinner).

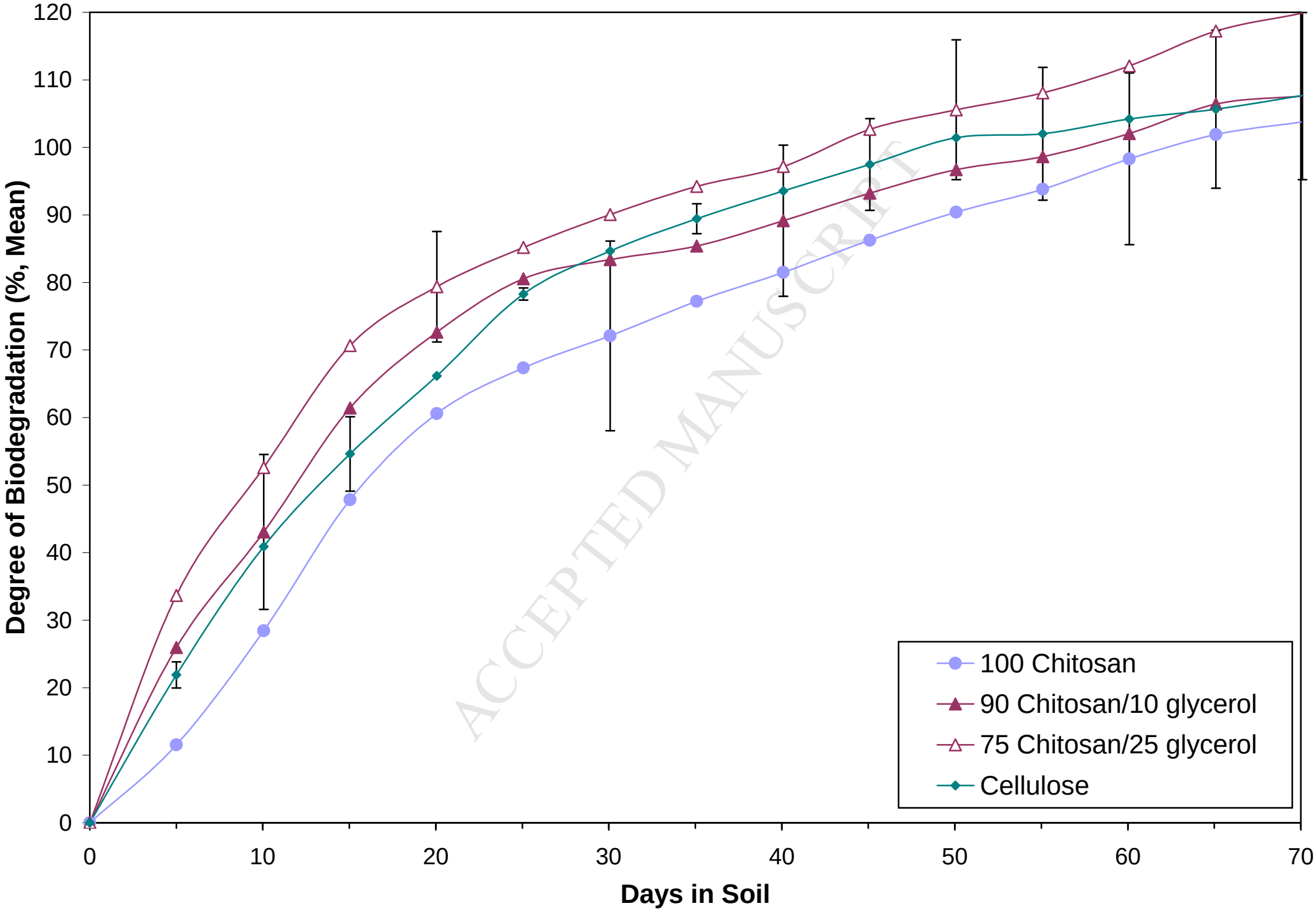
(A) Cellulose

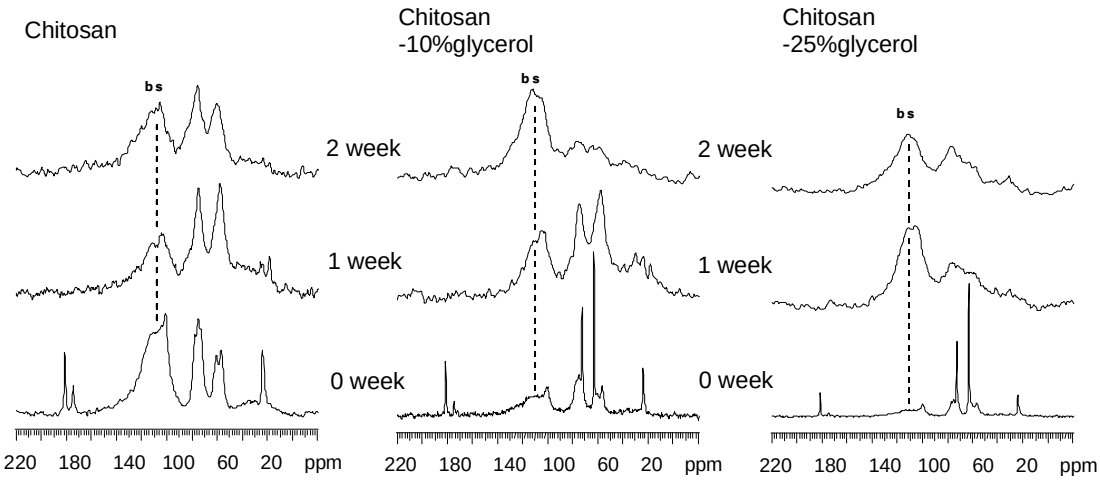
(B) 100 chitosan

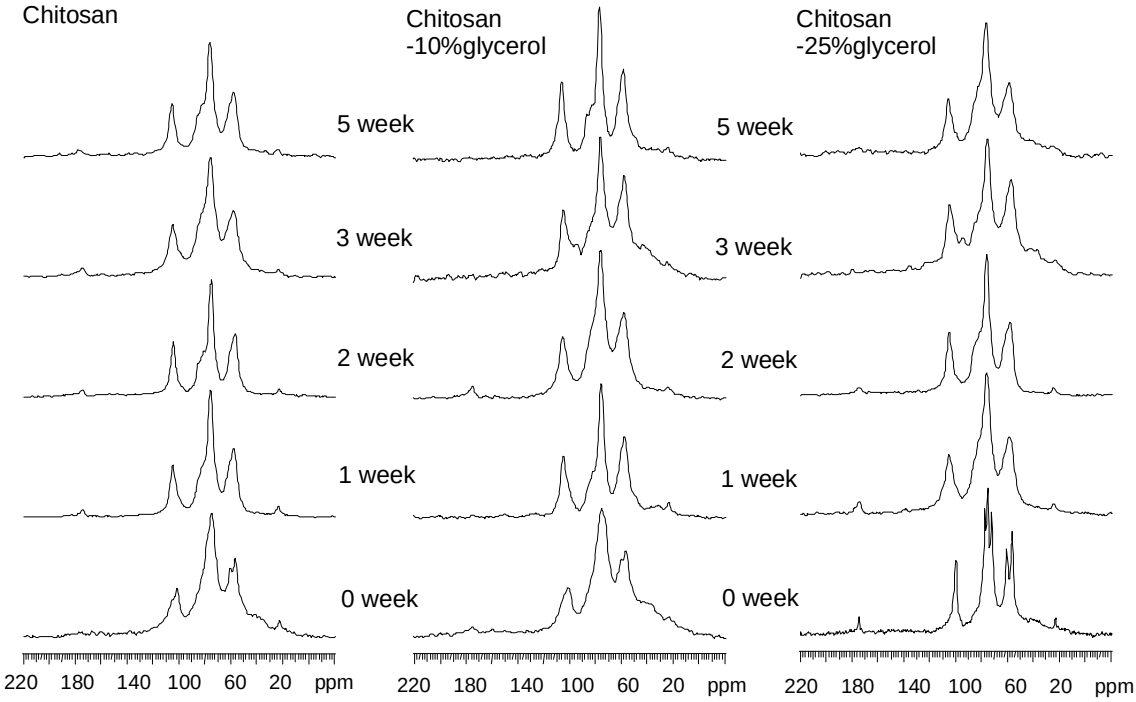
(C) 90 chitosan/10 glycerol

(D) 75 chitosan/25 glycerol









Cellulose

Neat chitosan

90 chitosan/10 glycerol

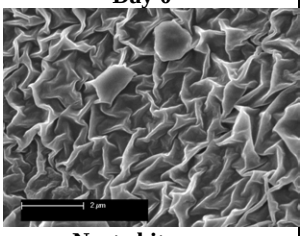
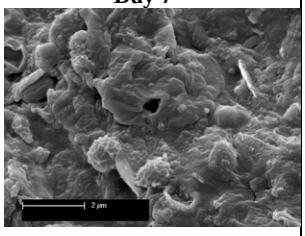
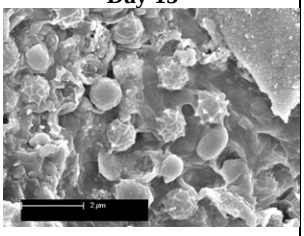
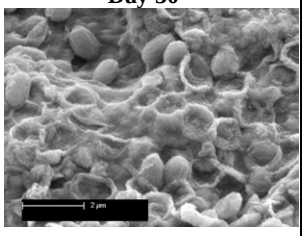
75 chitosan/25 glycerol

75 chitosan/25 glycerol/2.5 MMT

75 chitosan/25 glycerol/5 MMT

75chitosan/25 glycerol/modified 2.5 MMT

75 chitosan/25 glycerol/ modified 5 MMT

Day 0	Day 7	Day 15	Day 30
			
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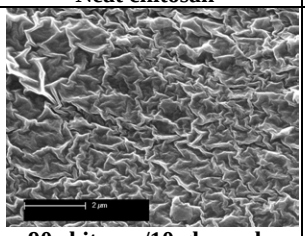	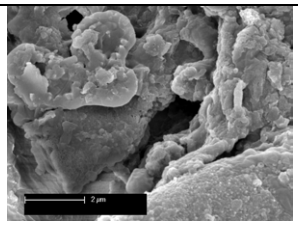	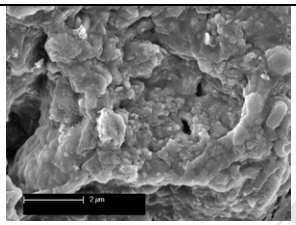	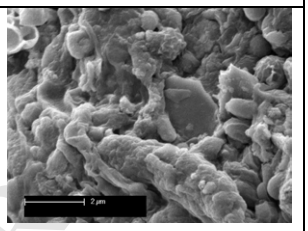
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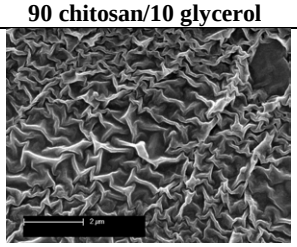	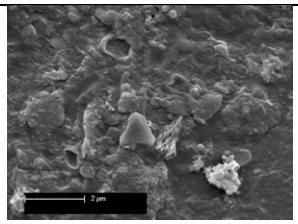	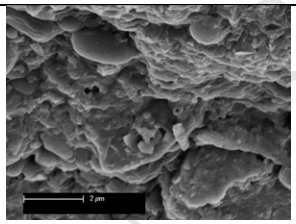	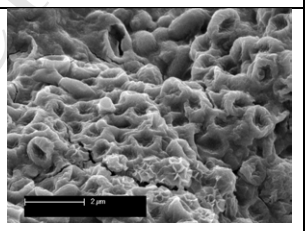
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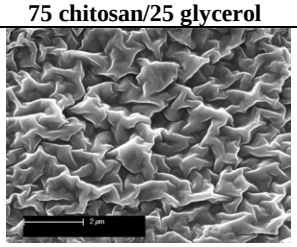	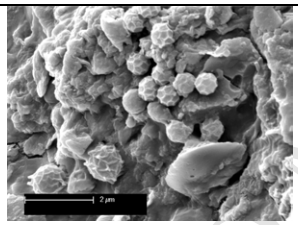	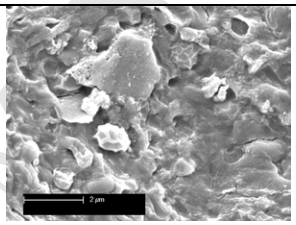	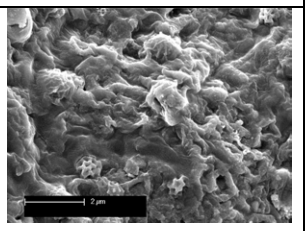
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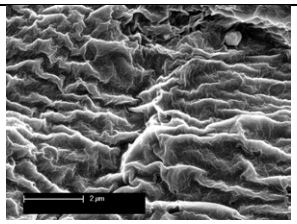	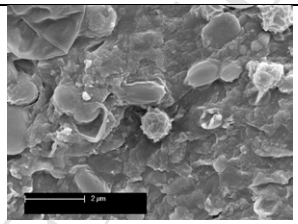	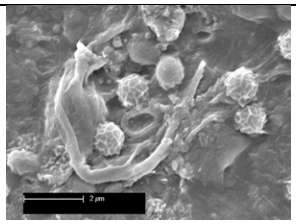	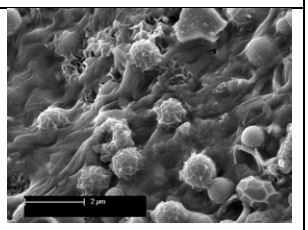
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